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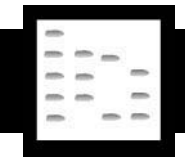
LIPIDS



Gas Chromatography

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Introduction

Natural lipids consist of complex mixtures of molecular species, which are found in association with cell membranes, lipoproteins and other subcellular structures. The composition differs among different cell and tissue types, reflecting the function of lipids in these body structures. Industrial and food products may be of plant, animal or synthetic origin. The gas chromatography (GC) of fatty acids by James and Martin in 1956 provided the first success in dealing with the complexity of the hydrolysis products of fats and oils. A few years later Kuksis and McCarthy developed methods for the resolution of intact triacylglycerols by high temperature GC.

Improved design of GC equipment, culminating in the development of reliable capillary columns, together with enzymic and chemical derivatization of samples now permits the separation of molecular species of all lipids on a routine basis. In modern analyses, conventional or high temperature GC serves as the final step in the multi-method resolution and quantification of individual components of a total lipid extract. The high molecular weight and low volatility, however, require constant vigilance in quantitative GC analysis of natural lipids.

Nonpolar Capillary Columns

Nonpolar capillary columns provide resolution based on the overall molecular weight of the lipid molecules. The nonpolar phases are typically polymethylsiloxane polymers with 5% phenyl groups. They are stable to 350°C or higher temperatures. Other nonpolar phases for GC are provided by certain hydrocarbons, which are limited to much lower temperatures and are operated isothermally.

Polarizable Capillary Columns

The methylsiloxane liquid phases containing 50–65% phenyl groups become polar as the temperature increases above 290°C, as indicated by the longer retention of the unsaturated compared to saturated triacylglycerols. Below this temperature the liquid phase is nonpolar, as indicated by the earlier elution of the unsaturated compared to saturated fatty acid trimethylsilyl (TMS) esters. The polarizable liquid phases are stable up to 360°C. Since these liquid phases possess high temperature stability, they are well suited for the resolution of molecular species of seed oil and milk fat triacylglycerols.

However, columns at least 25 m long are needed to provide sufficient resolving power for the separation of the saturated and unsaturated species of natural diacyl and triacylglycerols, and ceramides.

Polar Capillary Columns

Capillary columns containing polar and very polar liquid phases are utilized mainly for the separation of saturated and unsaturated fatty acids as the methyl esters. The more stable polar capillary columns can be programmed to 280°C and used for the separation of the TMS ethers of diacylglycerols and ceramides as well as low molecular weight triacylglycerols.

Detection and Quantification of GC Peaks

The principal detector for the GC of lipids is the flame ionization detector (FID), but electron-capture detectors are also used (e.g. for pentafluorobenzyl esters). A number of authors have evaluated the quantitative GC analysis of fatty acids and triacylglycerols. GC with online electron impact (EI) mass

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spectrometry (GC-EI-MS) yields largely qualitative information from which the structure of unknown molecules can nevertheless be deduced. However, both total ion current and single ion monitoring have been utilized for quantitative analyses using appropriate calibration curves.

Resolution of Neutral Lipids

Neutral lipids, including common triacylglycerols, are readily resolved according to their molecular weight or number of carbon atoms by high temperature GC. Mixtures of high and low molecular weight neutral lipids are best dealt with by temperature programming. These methods are also suitable for the GC of certain polar lipids provided the polar head groups have been removed or masked. The neutral lipid separations are usually performed on nonpolar liquid phases, but polarizable liquid phases of high temperature stability have also been employed for the separation of intact neutral lipids. In both instances some prefractionation of the lipid mixture is necessary for optimum analysis.

Isolation and Preparation of Derivatives

Usually this includes the removal of the polar lipids from the neutral compounds by thin-layer chromatography (TLC), high performance liquid chromatography (HPLC) or simple adsorbent cartridges. Before GC, the free functional groups of the lipids must be protected in order to avoid dehydration and to increase volatility, as well as to improve other GC properties. This is readily accomplished by preparing TMS derivatives; in special instances other derivatives may be prepared. Free carboxyl groups may be methylated with diazomethane and alcohol groups may be acetylated with acetic anhydride in the presence of other ester bonds. **Table 1** lists the more common reagents for derivatization of neutral lipids along with the reaction conditions. Polar lipids, such as glycerophospholipids, can be converted into neutral lipids by dephosphorylation. This can be readily accomplished by hydrolysis with phospholipase C (*Bacillus cereus*), which releases the phosphocholine moiety from phosphatidylcholine, lysophosphatidylcholine and sphingomyelin, and the phosphoethanolamine moiety of both diacyl and alkenylacylglycerophosphoethanolamines. The *B. cereus* enzyme also attacks the plasma inositol phosphatides to yield diacylglycerol moieties. Alternatively, the plasma phospholipids may be dephosphorylated by pyrolysis, acetolysis and silolysis, but the latter procedures lead to isomerization of the acylglycerols, incomplete conversion and partial destruction of the lipid samples.

Table 1 Preparation of derivatives for GC analysis of neutral lipids, fatty acids and prostanoids[a]

Reagent	*Ratio*	*Temperature*	*Time*	*Application*
Neutral lipids				
Pyridine/HMDS/TMCS	12/5/2	Ambient	0.5–1 h	Trimethylsilyl ethers of acyglycerols and sterols
t-BDMCS/imidazole/ dimethylformamide	1 : 2.5 in DMF	80°C	20 min	*tert*-Butyldimethylsilyl ethers of acylglycerols and sterols
Ac_2O/pyridine	1 : 10	80°C	1 h	Acetates of acylglycerols and sterols
PFB_2O/pyridine	1 : 10	80°C	1–2 h	Pentafluorobenzoates of acylglycerols and sterols
Fatty acids				
BF_3/MeOH	15%	Ambient	20 min	Methyl esters of free fatty acids
BF_3/MeOH	6–15%	60°C/reflux	2–10 min	Methyl esters of fats and oils
H_2SO_4/MeOH	6%	80°C/reflux	2 h	Methyl esters and dimethylacetals of fatty acids
HCl/MeOH	5%	60°C	0.5–2 h	Methyl esters of free and bound fatty acids
CH_2N_2/ether	Dilute	Ambient	5 min	Methyl esters of free fatty acids
KOH/MeOH/benzene	0.2–2 N	Ambient	0.5 min	Methyl esters of glyceryl esters
NaOH/MeOH	0.5–2 N	Refluxing	0.5–1 h	Methyl esters of steryl esters
2-NH_2-2-MePr (DMOX)		170°C	18 h	4,4-Dimethyloxazolines of unsaturated fatty acids
DEADMS/3-pyridyl carbinol		60°C	10 min, 10 min	Picolinyldimethylsilyl (PICSI) esters
$(TFA)_2O$/3-pyridyl carbinol	2 steps	50°C	1 h	Picolinyl esters of fatty acids
Oxalyl Cl/pyrrolidide	2 steps	Ambient	30 min	*N*-acyl pyrrolidides of fatty acids
Prostanoids				
PFBBr/*N*,*N*-DIPEtn/ Meoxamine/BSTFA/pyridine	3 steps	Ambient, 60°C, and 60°C, resp.	10 min, 16 h, 15 min	PFB/MO/TMS derivatives of prostanoids, thromboxanes, hepoxilins

[a]Abbreviations and experimental details are found in the text, in legends to figures and in references cited.

The dephosphorylated lipids are recovered by TLC or adsorbent cartridges and the exposed hydroxyl groups masked by acetylation or preparation of TMS derivatives. The diacyl, alkylacyl and alkenylacyl subclasses released from glycerophospholipids by phospholipase C can be readily resolved by normal-phase HPLC or TLC prior to the GC resolution of the molecular species of the *sn*-1,2-diradylglycerol moieties. Likewise, the method is suitable for the resolution of the molecular species of TMS ethers of the *sn*-1,2- and *sn*-2,3-diacylglycerol moieties of triacylglycerols derived by Grignard degradation, chiral-phase HPLC resolution as the dinitrophenylurethanes, and silolysis.

Total Neutral Lipid Profiling

Total neutral lipids are easily resolved into component lipid classes by GC on short (8–15 m) nonpolar capillary columns. Longer (25 m) polarizable capillary columns provide separations of molecular species. The components ranging from free fatty acids to triacylglycerols can be effectively quantified by tridecanoylglycerol added as an internal standard to the mixture prior to the GC analysis. **Table 2** lists the more common liquid phases for GC of neutral lipids along with the column conditions and selected applications.

Nonpolar capillary GLC **Figure 1** shows a GC separation of total lipids in plasma, following dephosphorylation and preparation of TMS derivatives, on a nonpolar capillary column along with that of a mixture of ceramides and monoacylglycerols released from the combined sphingomyelin and lysophosphatidylcholine fraction isolated by preliminary TLC. The separations on the nonpolar capillary column are limited to resolution by carbon number, although the unsaturated species are eluted slightly ahead of the saturates of the same carbon number. The ceramides (peaks 32–42) are eluted over the same temperature range as the diacylglycerols (peaks 34–40). GC separations similar to those obtained for the total plasma lipids are readily obtained for individual plasma lipoproteins and lymph chylomicrons, as well as for other total lipid extracts from natural sources that can be converted to neutral lipids. GC-MS of plasma total lipids provides confirmation of the peak identity. Mass chromatograms of characteristic fragment ions, retrieved from the total ion current by a computer, permit identification and quantification of overlapping molecular species of free fatty acids, their esters and amides, as well as free sterols and steryl esters.

Partial separation of saturated and unsaturated plasma cholesteryl esters has been reported on a nonpolar capillary column and identification confirmed by negative ammonia ionization mass spectrometry. GC-MS analysis of synthetic steryl esters by nonpolar capillary GLC and EI and chemical ionization has been reported. The GC separation of C_{27} sterols has

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Table 2 Commonly used liquid phases and columns for GC of neutral lipids[a]

Chemical composition	*Commercial names*	*Column dimensions*	*Type of separation (temperature programme)*	*Applications*
Methylsilicone	BP-1 (OV-1, SE-30, SP-2100)	12 m × 0.22 mm i.d.	Carbon number (200–350°C)	Cholesteryl esters
5% Phenyl, 95% methylsilicone	SE-54 (DB-5, HP-5, BP-5, OV-5[b])	8 m × 0.32 mm i.d.	Carbon number (200–350°C)	Triacylglycerols, steryl esters
65% Phenyl, 35% methylsilicone	RSL-300 (OV-22, Rtx-65-TG)	25 m × 0.25 mm i.d.	Carbon and double bond number (40–360°C)	Triacylglycerols, steryl esters, ceramides
Methylsilicone	SE-52 (DB-, SE-54)	26 m × 0.3 mm i.d.	Carbon number (200–350°C)	Triacylglycerols, steryl esters
100% Dimethylsilicone	OV-1 (SP-2100, BP-1, DB-1)	5 m × 0.32 mm i.d.	Carbon number (100–350°C)	Triacylglycerol core aldehydes
100% Dimethylsilicone	OV-1 (SE-30, BP-1, SP-2100)	15 m × 0.3 mm i.d.	Carbon number (200–350°C)	Triacyglycerol core aldehydes
68% Cyanopropyl, 32% dimethylsiloxane	Rtx-2330 (SP-2330, SP-2560)	15 m × 0.32 mm i.d.	Carbon and double bond number (250°C, isothermal)	Diradylglycerol TMS and TBDMS ethers
100% Dimethylsilicone	SE-30 (SP-2100, DB-1, BP-1)	12 m × 0.25 mm i.d.	Carbon number (40–350°C)	Diradylglycerol TMS and TBDMS ethers

[a]Abbreviations and experimental details are found in the text, in legends to figures and in references cited.
[b]Comparable liquid phases are given in brackets.
(Modified with permission from Restek Corporation (1998) *Chromatography Products*, International Version, pp. 544–545.)

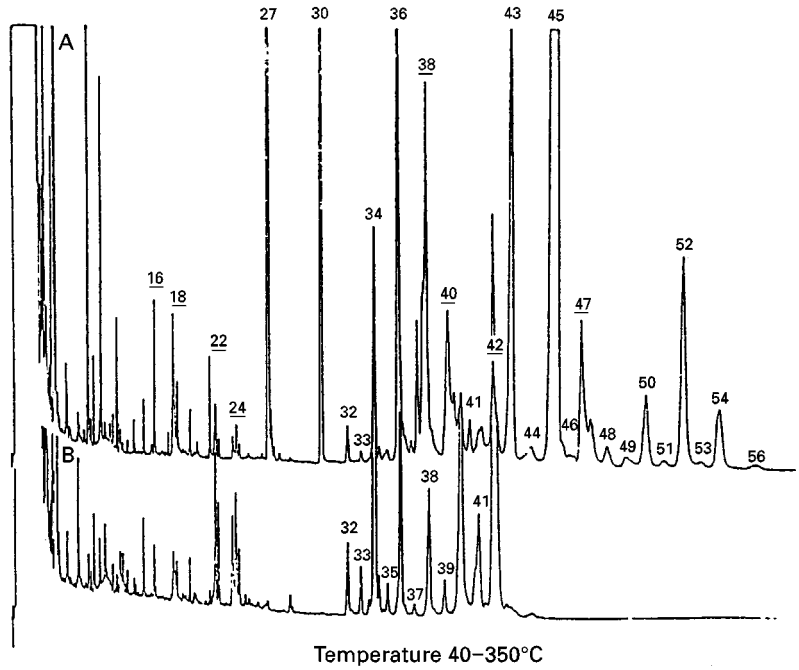

Figure 1 GC of total lipids of normal human plasma on a nonpolar capillary column. A, Total neutral lipids as obtained by dephosphorylation with phospholipase C; B, ceramides and monoacylglycerols released from sphingomyelin and lysophosphatidylcholine. Simplified peak identification: 16 and 18, free fatty acids; 22–24, monoacylglycerols; 27, free cholesterol; 30, tridecanoylglycerol (internal standard); 32–42, diacylglycerols and ceramides; 43–47, cholesteryl esters; 48–56, triacylglycerols. GC conditions: column, 5 m × 0.25 mm i.d., 100% dimethylsilicone (SP-2100, Supelco); temperature, programmed, 170–350°C at 8°C min^{-1} with hydrogen as carrier gas. (Reproduced with permission from Myher JJ and Kuksis A (1984) Determination of plasma total lipid profiles by capillary gas liquid chromatography. *Journal of Biochemical and Biophysical Methods* 10: 13–23.)

been reviewed and the retention times tabulated on DB-5 and CP-WAX columns for a large number of compounds as the TMS derivatives in relation to 5α-cholestane.

Polarizable capillary GLC **Figure 2** compares the plasma total lipid profiles as obtained by GC on (A) nonpolar and (B) polarizable capillary columns. The nonpolar column yields prominent peaks for free cholesterol, diacylgycerols and ceramides, cholesteryl esters and triacylglycerols. The lipid ester classes are resolved according to the total number of carbons. The polarizable column permits a separation of the glycerolipids and cholesteryl esters on the basis of both total carbon and double bond number. There is an extensive overlap among the molecular species of the diacylglycerols and ceramides but the cholesteryl esters are well resolved from each other and from the triacylglycerols of both higher and lower molecular weight. Characteristic lipid profiles are also obtained for the individual plasma lipoprotein classes. Cholesteryl arachidonate suffered some degradation and is incompletely recovered. The plasma triacylglycerols appear to be fully recovered, except for the more highly unsaturated long chain (56:4–66:18) species, which are only partially recovered.

Molecular Species of Diradylglycerols and Ceramides

The early GC analyses of molecular species of diacylglycerols were performed on nonpolar packed or capillary columns following a preliminary resolution based on unsaturation by argentation TLC. Polarizable capillary columns provide an improved resolution of the molecular species of diacylglycerols and especially of ceramides. **Figure 3** compares the order of peak elution of diacylglycerols (partial Grignard deacylation products of lard triacylglycerols) and the ceramide moieties of plasma sphingomyelin. The diacylglycerol and ceramide peaks are eluted in order of increasing number of double bonds within a carbon number of each lipid class. Thus, the diacylglycerol 16:0–18:1 is eluted ahead of 16:0–18:2 and 18:0–18:1 is eluted ahead of 18:0–18:2, but 18:0–18:2 tends to overlap with 16:0–20:4. The ceramide d18:1–16:0 elutes earlier than the diacylglycerol 16:0/16:0 on the polarizable column, whereas on nonpolar columns they overlap.

The molecular species of diacylglycerols are best resolved by GC on polar capillary columns similar to those used for separation of fatty acid methyl esters. These columns provide especially detailed analyses of

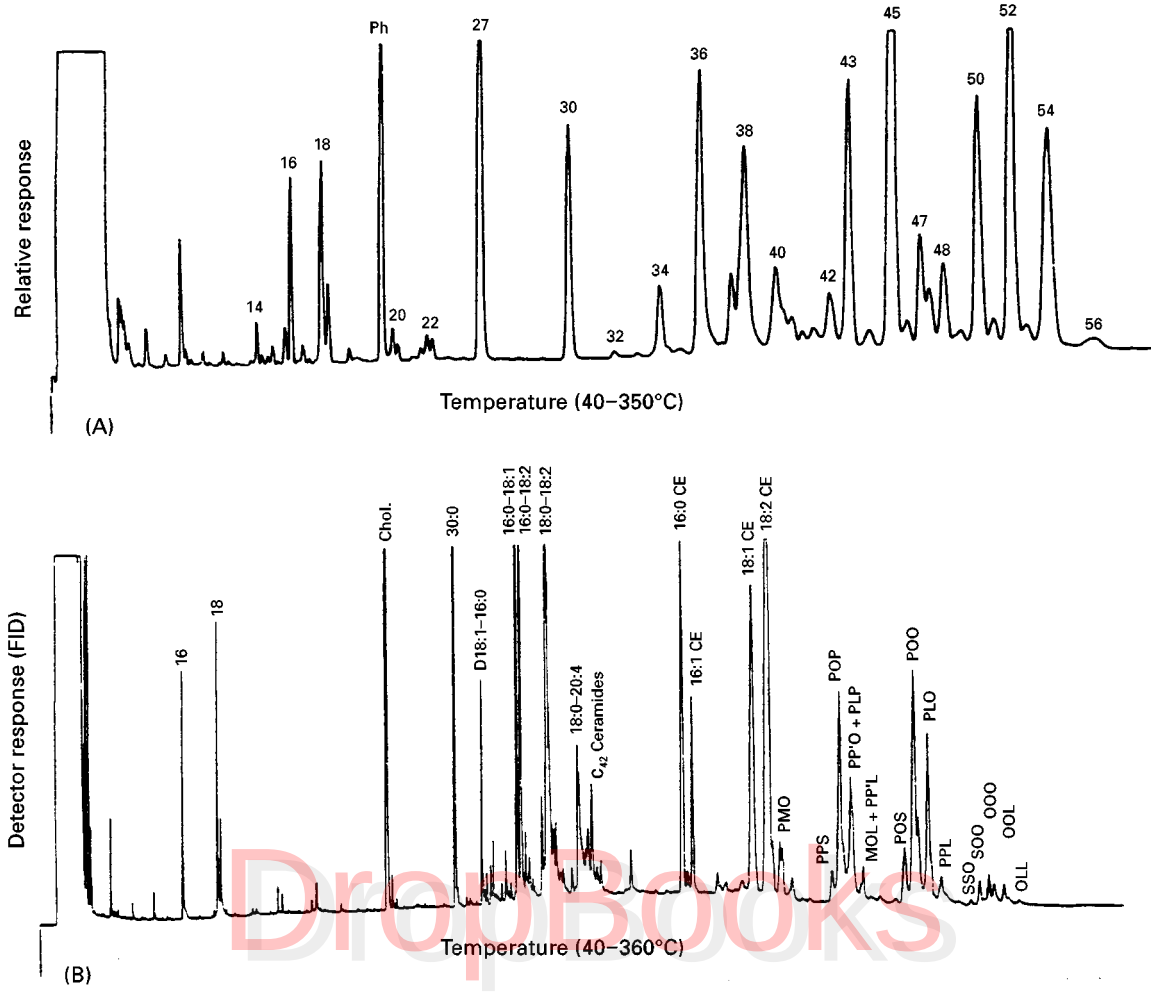

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Figure 2 GC profiles of plasma total lipids on (A) nonpolar and (B) polarizable capillary columns. Peak identification: (A) as given in Figure 1; Ph, TMS ester of phthalic acid; (B) as given in the figure; L, linoleic; M, myristic; O, oleic; P, palmitic; S, stearic acids. GC conditions: (A) column, 5 m × 0.25 mm i.d., dimethylsilicone (SP-2100, Supelco); temperature, programmed, 40–350°C at 8°C min^{-1} with hydrogen as carrier; (B) column, 25 m × 0.25 mm i.d., 65% phenylmethylsilicone (OV-22, Quadrex); temperature, programmed, 40–360°C at 50°C min^{-1} to 150°C, then 10°C min^{-1} to 310°C, and 2°C min^{-1} to 360°C with hydrogen as carrier gas. (Reproduced with permission from Kuksis A, Myher JJ and Geher K (1993) Quantitation of plasma lipids by gas liquid chromatography on high temperature polarizable capillary columns. *Journal of Lipid Research* 34: 1029–1038.)

the TMS ethers of the diradylglycerol moieties of glycerophospholipids and triacylglycerols. **Figure 4** shows the resolution of the molecular species of the diacyl, alkylacyl and alkenylacyl subclasses of human plasma ethanolamine glycerophospholipids as obtained by polar capillary GC of the TMS ethers of the derived diradylglycerols. There are marked differences in the distribution of the chain length and unsaturation among the three subclasses of the plasma ethanolamine glycerophospholipids as anticipated from the fatty acid and diradylglycerol carbon number distribution. Polar capillary GC resolution of molecular species of the *sn*-1,2- and *sn*-2,3-diacylglycerols has been extensively utilized in structural analyses of triacylglycerols. Tables have been compiled of GC retention factors of diradylglycerol TMS ethers on polar capillary columns.

Molecular Species of Triacylglycerols

GC is well suited for the separation of the molecular species of triacylglycerols. Nonpolar columns provided essentially carbon number or molecular weight resolution, while the polarizable liquid phases give effective separations of molecular species of saturated and unsaturated triacylglycerols. Polar capillary columns also provide separations based on both carbon and double bond number, but are limited to low molecular weight triacylglycerol mixtures.

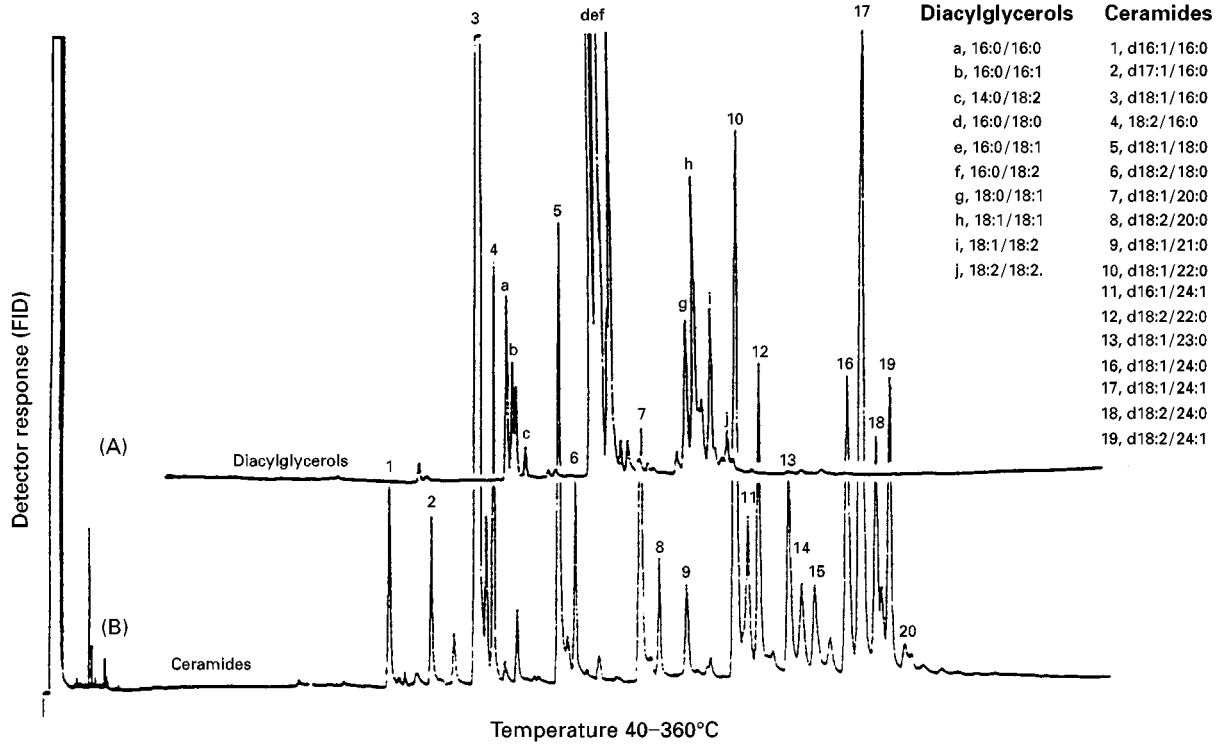

Figure 3 GC profiles of (A) common diacylglycerols and (B) the ceramides of plasma sphingomyelin as obtained on a polarizable capillary column. Peak identification is as given in figure. GC conditions are as given in Figure 2B. (Reproduced with permission from Kuksis A, Myher JJ and Geher K (1993) Quantitation of plasma lipids by gas liquid chromatography on high temperature polarizable capillary columns. *Journal of Lipid Research* 34: 1029–1038.)

Nonpolar liquid phases The first GC resolutions of molecular species of triacylglycerols were obtained on nonpolar packed columns of short length. **Figure 5** compares the separation of butteroil triacylglycerols obtained on a nonpolar packed column and on a capillary column. Both columns yield essentially carbon number resolution, although the capillary column shows some peak splitting due to separation of saturated and unsaturated species within a carbon number. A combination argentation TLC or HPLC with nonpolar GC separation is required for a more extensive separation of molecular species.

Nonpolar capillary GC columns have been effectively utilized for resolution of the reduction products of ozonized lard, rapeseed and palm oil triacylglycerols, as well as the reduction products of ozonized partially hydrogenated soybean oil. **Figure 6** shows the elution profile on a nonpolar column of the triacylglycerols of soyabean oil after reductive ozonolysis. The peaks are identified by the triplets of the fatty acids: palmitic (P), stearic (S) and the C_9 aldehyde (U) of the unsaturated fatty acid.

Polarizable liquid phases The usefulness of the polarizable liquid phases for the separation of saturated and unsaturated triacylglycerols was discovered by Geeraert and Sandra in 1984. **Figure 7** shows the resolution of the molecular species of butteroil triacylglycerols. Peak identification presents a problem because of extensive resolution of isobaric species. However, preliminary resolution by argentation TLC in combination with GC-MS enabled the pattern of elution to be established. At the present time, the polarizable capillary column provides the most complete GC resolution of the molecular species of triacylglycerols.

The resolution of triacylglycerols seen in the GC-MS profiles is somewhat reduced in comparison to GC-FID. The reduced resolution complicates post-column processing of the GC-MS data due to lack of discrete peaks in parts of the total ion chromatogram. GC-MS quantification of milk fat triacylglycerols is improved by employing the Biller–Biemann enhancement technique to produce mass-resolved total ion chromatograms. The Biller–Biemann enhancement algorithm examines each mass that appears in successive scans in the TLC to detect masses that are rising and falling in intensity.

Polar liquid phases The polar capillary columns commonly employed for fatty acid methyl ester separation can resolve low molecular weight

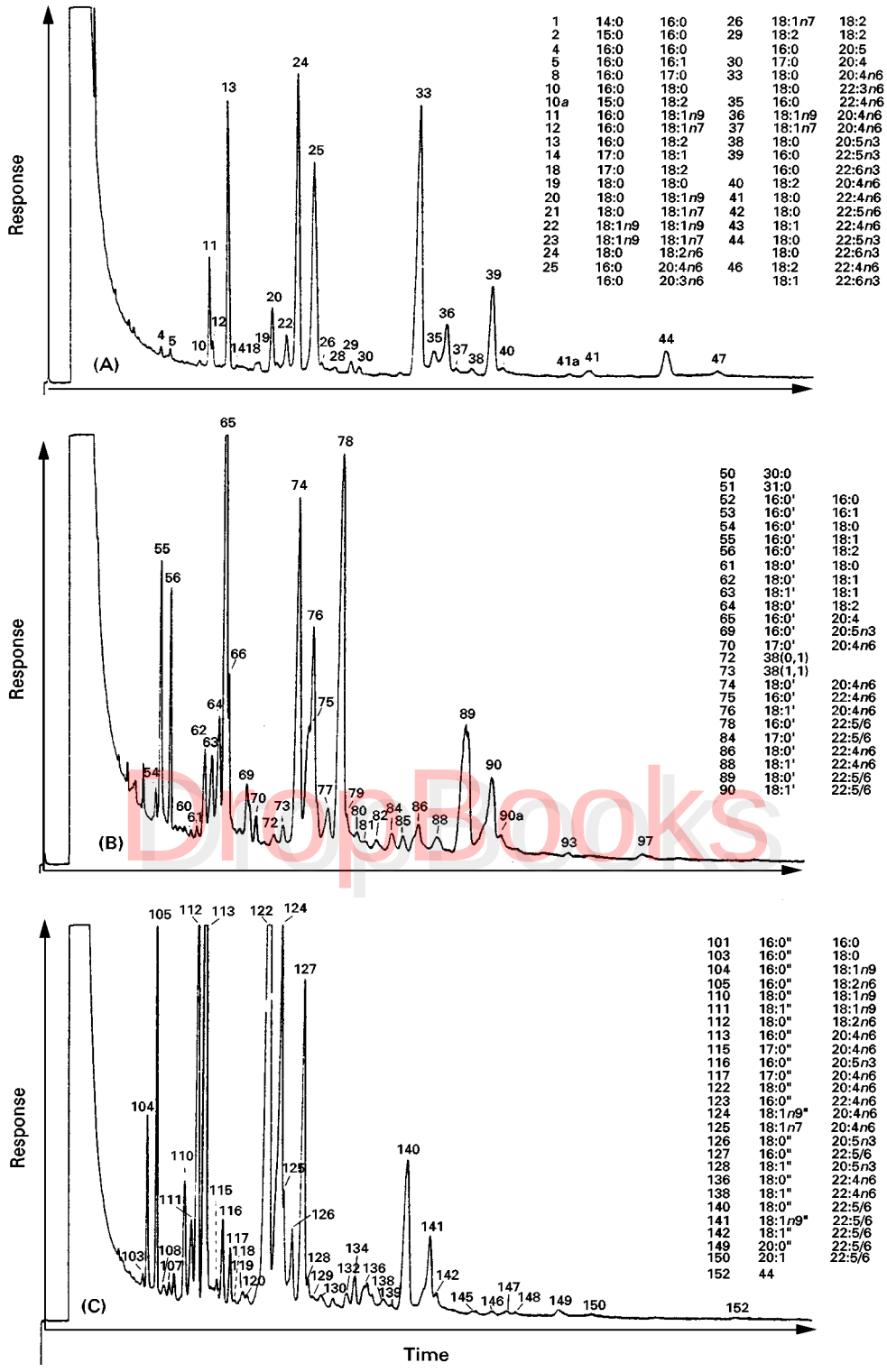

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Figure 4 GC profiles of the TMS ethers diradylglycerol moieties of human plasma diradylglycerophosphoethanolamines (GPE). Peaks are identified in the figures. (A) Alkylacyl GPE; (B) alkenylacyl GPE; (C) diacyl GPE. Peak identification is given in the figures. GC conditions: column, 15 m × 0.32 mm, i.d. 68% cyanopropyl/32% dimethylsiloxane (RTx 2330, Restek); temperature, isothermal, 25°C with hydrogen as carrier gas. (Reproduced with permission from Myher JJ, Kuksis A and Pind S (1989) Molecular species of glycerophospholipids and sphingomyelins of human plasma: comparison to red blood cells. *Lipids* 24: 408–418).

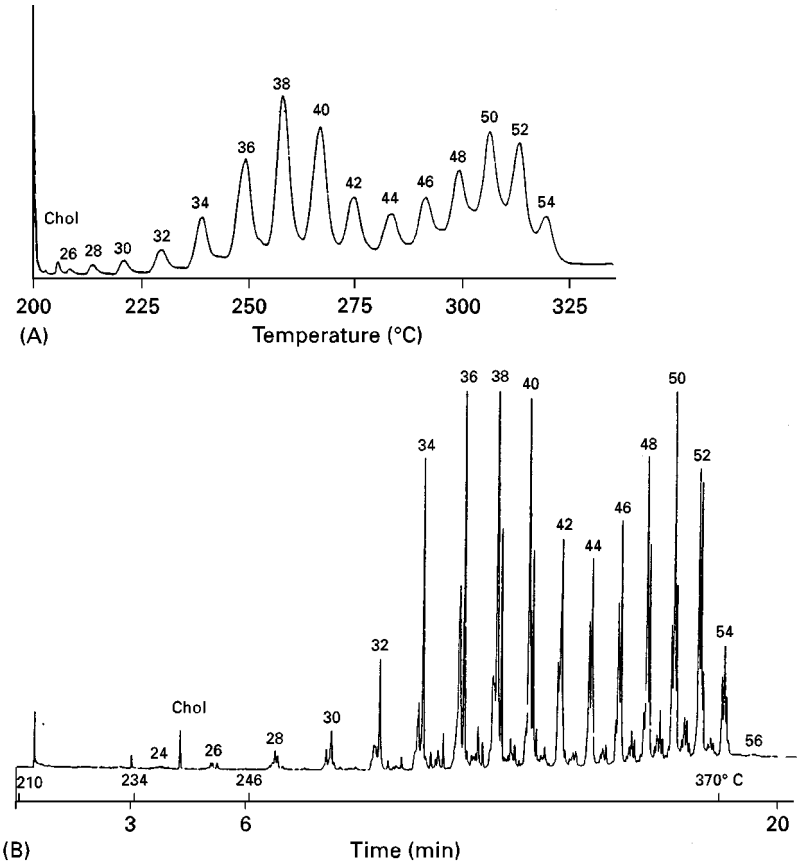

Figure 5 GC profiles of butteroil triacylglycerols as obtained on nonpolar columns. (A) Packed column; (B) capillary column. Peaks are identified by total acyl carbon as shown in the figures. GC conditions: (A) packed column, 50 × 0.25 cm, i.d. 2.5% SE-30, Applied Science; temperature, linearly programmed from 200 to 325°C with nitrogen as a carrier gas (Reproduced with permission from Kuksis A and McCarthy MJ (1962) *Canadian Journal of Biochemistry and Physiology* 40: 679–685.) (B) Capillary column, 25 m × 0.25 mm i.d., methyl silicone (OV-1, Ohio Valley); temperature, programmed from 210 to 370°C in 20 min with hydrogen as a carrier gas. (Reproduced with permission from Geeraert, 1987.)

triacylglycerols and the diacetates of monoacylglycerols containing saturated and unsaturated fatty acids. Conventional polar capillary columns are not sufficiently stable at the high temperatures needed to elute long chain triacylglycerols.

Resolution of Fatty Acids

Fatty acids constitute the most extensively investigated class of lipids for GC separation, identification and quantification. Although the routines for the common fatty acids are well established, determination of minor fatty acids, including branched-chain and positional and configurational isomers, requires specialized approaches. Other problems arise from the presence of oxygenated functional groups in the fatty chains. Many of these fatty acids have been successfully identified by preparing nitrogenous derivatives in combination with GC-MS.

Isolation and Preparation of Derivatives

Proper isolation and preparation of derivatives are essential for both identification and quantification of fatty acids. The common procedures for isolating fatty acids consist of extracting lipids from biological materials, saponification and esterification of the recovered acids. Underivatized volatile fatty acids are difficult to quantify by GC because these highly polar compounds form hydrogen bonds that interact with the active sites on the column coatings and cause peak tailing and ghosting due to dimerization. These undesirable effects are avoided by converting the fatty acids into methyl esters or other volatile derivatives. Specially developed columns, however, may avoid both tailing and ghosting.

Preparation of methyl esters Table 1 also lists the more popular methods of derivatization of the common fatty acids for GC analysis. Formation of fatty

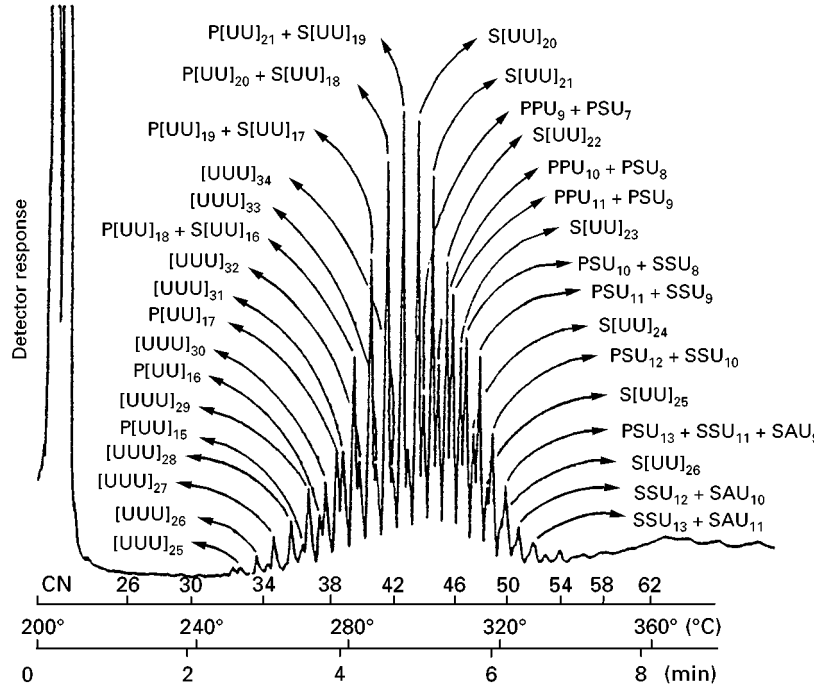

Figure 6 Non-polar capillary GC profile of the triacylglycerols of soybean oil after reductive ozonolysis. Peak identification is as given in figure: P, palmitic; S, stearic; U_n, unsaturated fatty acid residues. GC conditions: column 15 m × 0.32 mm i.d. 100% methylsilicone (OV-1, Ohio Valley); temperature, 200°C (0.15 min) isothermal then 20°C min^{-1} to 360°C with hydrogen as carrier gas. (Reproduced with permission from Geeraert E (1985) In: Sandra P (ed.) *Sample Introduction in Capillary Gas Chromatography*, vol. 1, pp. 133–158. Heidelberg: Alfred Hüetig Verlag.

acid methyl esters is usually accomplished in the presence of acid or alkaline catalysts. Acidic catalysts transesterify glycerolipids and other complex lipids as well as esterify any free fatty acids in the presence of methanol. Inclusion of butylated hydroxytoluene as an antioxidant in the transmethylation solution results in the formation of methoxy butylated hydroxytoluene, which emerges in the 14:0–16:0 fatty

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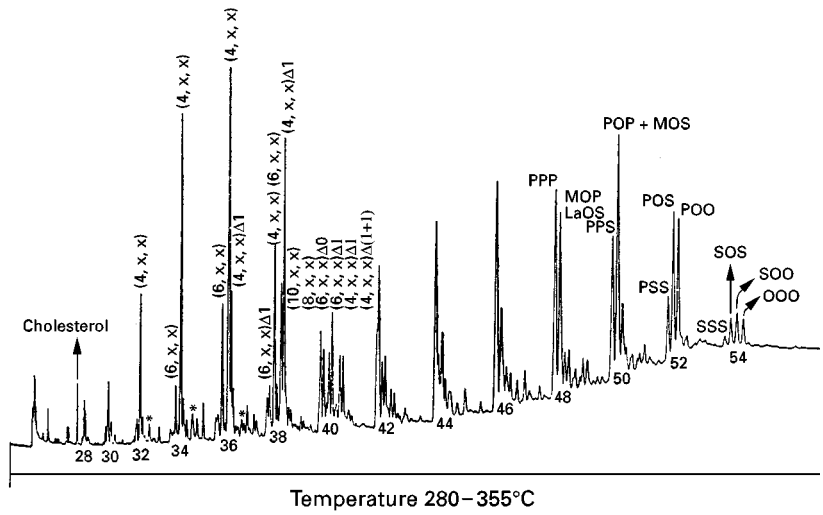

Figure 7 Polarizable capillary GC profile of butteroil triacylglycerols. Peak identification is as given in figure: P, palmitic; O, oleic; S, stearic; M, myristic acids; 28–54, total acyl carbon numbers; short chain triacylglycerols are shown as combinations with major long chain acids (x) of different degrees of unsaturatation ($\Delta 0 - \Delta 1 + 1$) as identified elsewhere (Myher JJ, Kuksis A, Marai L and Sandra P (1988) *Journal of Chromatography* 452: 93–118.) GC conditions: column, 25 m × 0.25 mm i.d., 50% phenylmethylsilicone (RSL-300, supplied by Sandra P); temperature, linearly programmed from 280 to 355°C over 30 min with hydrogen as the carrier gas. *Major acetate peaks. (Reproduced with permission from Geeraert E and Sandra P (1987) Capillary GC of triglycerides in fats and oils using a high temperature phenylmethylsilicone stationary phase. Part II. The analysis of chocolate fats. *Journal of the American Oil Chemist's Society* 64: 100–105.)

acid elution region. Phthalate esters have also been detected among plasma lipids, even when prepared in the absence of external contamination. The precise point of elution relative to fatty acid derivatives is dependent on the nature of the phthalate ester and the stationary phase, but typically it is in the same range as the C_{18}–C_{22} fatty acids. In many instances it is convenient to perform the acid transmethylation *in situ*, e.g. in the presence of silica gel scrapings from a TLC plate.

Acid-catalysed transmethylation also leads to significant dehydration of the sterol moiety and degradation of conjugated fatty acids. The peaks formed during acid-catalysed methylation were identified as positional allylic methoxy isomers of 18:1 by GC-MS. Re-evaluation of the H_2SO_4–isopropanol reaction showed just as extensive isomerization of conjugated dienes as the HCl–MeOH method.

Alkaline catalysts transesterify neutral lipid esters in anhydrous methanol much faster (a few seconds to a few minutes), but they are unable to esterify free fatty acids, which constitutes a serious shortcoming. Furthermore, *N*-acyl lipids are not methylated. The presence of water leads to saponification. Alkaline reagents dissolve silica gel and cannot be employed with gel scrapings from TLC plates. Diazomethane can be prepared in ether solution by the action of alkali on a nitrosamide (e.g. *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide) in the presence of alcohol. This reagent is commercially available as Diazald (Aldrich Chemical Co). Diazomethane is used for preparation of fatty acid methyl esters from lipids that have been first saponified. It has the disadvantages that it is highly toxic, explosive and likely to cause specific sensitivity.

Preparation of other derivatives Table 1 also lists a selection of reagents for derivatization of oxygenated fatty acids and prostanoids. Monohydroperoxy fatty acids are reduced to hydroxy acids after reaction for 1 h at room temperature with triphenylphosphine in diethyl ether prior to GC analysis of the methyl ester TMS ethers. The monohydroxy fatty acid methyl esters are hydrogenated for 5 min at room temperature in the presence of rhodium on alumina in methanol. They are converted to their TMS ether derivatives by reaction with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide for 30 min at room temperature. *tert*-Butyldimethylsilyl ethers of secondary hydroxy fatty acid methyl esters are prepared by dissolving the sample in a mixture of dry toluene, dimethylformamide and pyridine, and adding *N*-*tert*-butyldimethylsilylimidazole and *N*-methyl-*tert*-butyldimethylsilyltrifluoroacetamide containing 1% *tert*-butyldimethylsilylchloride (TBDMS).

Of the many different derivatives described in the literature for GC analysis of prostaglandins, the pentafluorobenzoate–methoxime–TMS (PFB–MO–TMS) derivatives yield the most satisfactory results in terms of GC properties, electron-capture detection and MS response and stability. These derivatives show single well-shaped peaks for each compound, except for prostaglandin E_2–PFB–MO–TMS, whose *syn-anti* isomers can be seen as well-separated peaks.

Normal oxygenated and nonoxygenated fatty acids have been converted into nitrogenous derivatives for structural analyses by GC-MS. The picolinyl esters are prepared from the fatty acids by first converting them into acid chlorides by dissolving in thionyl chloride. The acid chlorides are then treated with a dilute solution of 3-pyridylcarbinol in acetonitrile. The picolinyldimethylsilyl derivatives of fatty alcohols are prepared by dissolving the alcohol in dry pyridine, adding diethylaminodimethylsilyl-3-pyridylcarbinol and heating at 60°C for 10 min. The picolinyl esters of the epoxides are prepared by dissolving the epoxide in dry methylene chloride and adding a freshly prepared solution of 1,1′-carbonyldiimidazole followed by 3-pyridylcarbinol solution of triethylamine. The preparation of picolinyl esters via fatty acid chlorides cannot be used for derivatization of the epoxides because of their instability in acidic solutions.

The pyrrolidides are prepared from the lipid ester. The sample is heated with an excess of pyrrolidine in the presence of acetic acid for 30 min at 100°C. 4,4-Dimethyloxazoline derivatives also have useful properties for locating functional groups by MS, but quantitative preparation requires heating the methyl esters with 2-amino-2-methylpropanol at 180°C overnight.

Separation of Saturated and Unsaturated Fatty Acids

Table 3 lists the more popular liquid phases for the separation of saturated and unsaturated fatty acids and prostanoids. The Carbowax phases based on polyethylene glycol appear to provide the best general-purpose columns.

Normal chain fatty acids and dimethylacetals For equivalent chain length (ECL) measurements, the separations of saturated and unsaturated fatty acids are performed isothermally. Isothermal analyses give good ECL values and these are remarkably consistent for the bonded polyglycol columns. Extensive tables of ECL values have been compiled for the methyl ester derivatives of natural fatty acids on silicone,

Table 3 Common liquid phases for capillary GC of fatty acids and their oxygenated derivatives[a]

Chemical composition	*Commercial names*	*Column dimensions*	*Type of separation (temperature programme)*	*Applications*
90% bis-Cyanopropyl/ 10% cyanopropylphenylsilicone	SP-2380 (OV-275/CP-Sil-88)	15 m × 0.32 mm i.d.	Carbon and double bond number (260–270°C)	Short chain triacylglycerols of butteroil distillates
Cross-bonded polyethylene glycol	Supelcowax-10 (Carbowax)	30 m × 0.25 mm i.d.	Saturates and polyunsaturates (185–220°C)	Fish oil fatty acids
Cross-bonded polyethylene glycol	Stabilwax (Carbowax CP 51)	60 m × 0.25 mm i.d.	Saturated and unsaturated esters (212°C, isothermal)	Seed oils and fats, marine oils
90% bis-cyanopropyl/10% cyanopropylphenyl	Rtx-2330 (CP-Sil-88, BPX-70)[b]	100 m × 0.25 mm i.d.	Δ^5-Δ^{16}-isomers of *trans*-18:1 (160°C, isothermal)	*Trans*-18:1 of beef tallow and human milk
Biscyanopropyl, 68%/ dimethylsilicone, 32%	SP-2380 (BPX-70/CP-Sil-84)	50 m × 0.25 mm	Cyclic fatty acid methyl esters (180°C, isothermal)	Hydrogenated cyclic fatty acid monomers from vegetable oils
14% Cyanopropylphenyl/ 86% polysiloxane	CP Sil 19 (DB-1701)	25 m × 0.25 mm	Methyl ethers of isomeric hydroxy fatty acids (100–250°C)	Hydrogenated hydroperoxides of plasma lipids
2% Dimethylsilicone	OV-101	0.4 m × 3 mm, packed	Carbon number (315–330°C, isothermal)	TMS ethers of mycolic acids
95% Dimethyl/5% phenyl silicone	SE-54 (HP-5/ DB5/CP-Sil-8)	18 m × 0.28 mm	Double bond location (70–240°C)	Oxazoline derivatives of fish oil fatty acids
Dimethylpolysiloxane	DB-1 (SE-30/OV-1)	30 m × 0.25 mm	Epoxide location (200–250°C)	3-Pyridinylmethyl esters of epoxides
Dimethylpolysiloxane	DB-1 (SE-30/OV-1)	20 m × 0.25 mm	Methoxy PFB esters (100–310°C)	Urinary prostanoids
86% Dimethyl/14% cyano propylphenyl polysiloxane	DB-1701 (DB-5, CP-Sil 19CB)	15 m × 0.2 mm	F_2-isoprostane TMS ethers and PFB esters (190–300°C)	Arachidonic acid peroxidation products
86% Dimethyl/14% cyano propylphenyl polysiloxane	DB-1701 (DB-5, CP-Sil 19CB)	30 m × 0.25 mm	Methoxy PFB esters (18–300°C)	Eicosanoids in blood

[a]Abbreviations and details of applications are given in the text, in legends to figures and in references cited.
[b]Comparable liquid phases are given in brackets.
(Modified with permission from Restek Corporation (1998) *Chromatography Products*, International Version, pp. 544–545).

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Carbowax, Silar 5CP and CP-Sil 84 columns. **Figure 8** shows the resolution of the fatty acid methyl esters of menhaden oil on a 30 m polyethylene glycol (Famewax, Restek) column using temperature programming (190–225°C). This liquid phase is stable to 250°C. The elution order of the complex polyunsaturated fatty acid methyl esters is comparable to that obtained on other Carbowax columns.

Acid-catalysed transmethylation leads to conversion of vinyl ethers (when present) into dimethylacetals, which are eluted ahead of the fatty acids of corresponding carbon number. **Figure 9** shows the resolution of the fatty acid methyl esters and dimethylacetals derived from human erythrocyte membranes on a deactivated cyanopropylsiloxane column. Although the separation of many of the dimethylacetals and the methyl esters is incomplete, it is still possible to obtain a good indication of the relative amounts of plasmalogens that may be present in the sample. Resolution of the major dimethylacetals and methyl esters on cyanopropylsiloxane columns has been obtained of one-third the length used by earlier workers. Prior to GC, the fatty acid methyl esters and dimethylacetals are resolved by normal-phase HPLC.

Identification of very long chain fatty acids An extensive series of long chain polyunsaturated fatty acids has been shown to occur in phosphatidylcholines of the vertebrate retina, where they make up a homologous series of even carbon polyenes having up to 36 carbon atoms. A detailed study has been made of these acids in bovine retina, where they were shown to belong to the same families as well-known fatty acids of the *n*-6 and *n*-3 series like arachidonate (20:4*n*-6), docosapentaenoate (22:5*n*-3) and docosa-

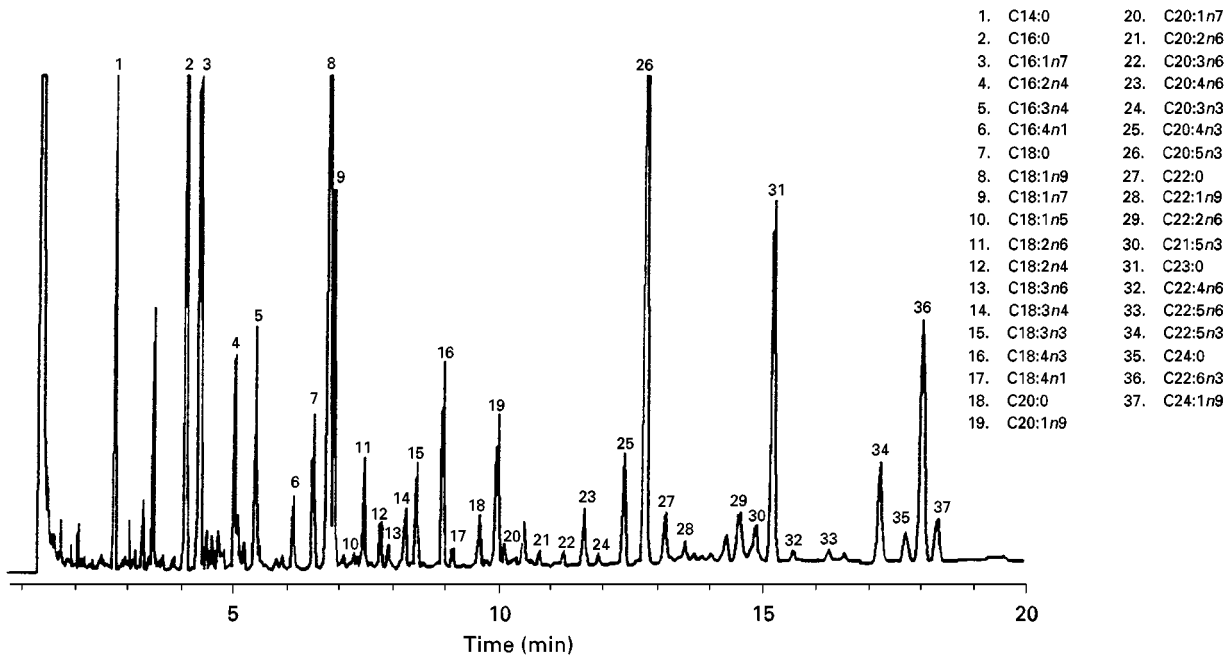

Figure 8 Polar capillary GC of fatty acid methyl ethers of menhaden oil. Peaks are identified in the figure. GC conditions: column, 30 m × 0.25 mm i.d., 0.25 μm cross-bonded polyethylene glycol (Famewax, Restek); temperature, 190°C (hold 4 min) then 4°C min^{-1} to 225°C; carrier gas, helium; split injection 1 : 50 (Reproduced with permission from Restek Corporation (1998) *Chromatography Products*, International Version, p. 447).

hexaenoate (22:6*n*-3). The fatty acids were analysed by temperature-programmed GC using glass columns packed with 15% OV-225. The position of the double bonds in the long chain polyenes was determined by means of oxidative ozonolysis.

Separation of short chain fatty acids Because of their high volatility, the short chain fatty acids create difficulties in GC separation and quantification when present in mixtures with long chain acids. The volatility can be effectively reduced by preparing higher molecular weight esters (propionyl or butyl esters) which also provide a more comparable response in the FID. It also permits a clear resolution of the short chain fatty acids from the solvent front and identification and quantification of the short chain acids in the presence of long chain acids. Recently developed high speed capillary columns (20 m × 0.10 mm i.d., 0.10 μm film thickness DB-WAX or DB-225, Restek) have been found to give an effective separation and quantification of both short and long chain fatty acids as methyl esters. A total of 37 fatty acids can be analysed in less than 15 min.

Separation of Oxygenated Fatty Acids and Prostanoids

Oxygenated fatty acids occur in nature as a result of enzymatic and nonenzymatic oxidation, and because of their physiological activity there is much interest in their analysis.

Fatty acid hydroperoxides are labile key intermediates in plant and mammalian lipid metabolism, acting as precursors of a variety of lipid-derived mediators such as prostaglandins and leukotrienes. Until recently, these analyses were usually done by GC, but now reversed-phase HPLC has been shown to have advantages for the analysis of the thermo-labile oxygenated derivatives of the fatty acids.

Hydroxides, epoxides, hydroperoxides and isoprostanes A 30 m nonpolar methylsiloxane column was used to measure the ECLs of hydrogenated derivatives of isomeric hydroxydocosahexaenoates. Mass spectra of hydrogenated compounds indicated the presence of hydroxyl groups at carbons 20, 16, 17, 13, 14, 10, 11, 7, 8 and 4. The isomers were apparently racemic mixtures.

GC-MS was used to identify the arachidonate epoxides/diols. The epoxide regioisomers of arachidonic acid as the methyl esters overlapped on nonpolar capillary columns. The isomeric epoxides were identified as their hydrolysis products, the dihydroxyeicosatrienoic (DHET) acids, in the form of the PFB ester derivatives. The four regioisomers were not resolved by GC as PFB, TMS, TBDMS or Me esters. However, after being hydrolysed to the dihydroxyeicosatrienoic acids, three of the four regio-

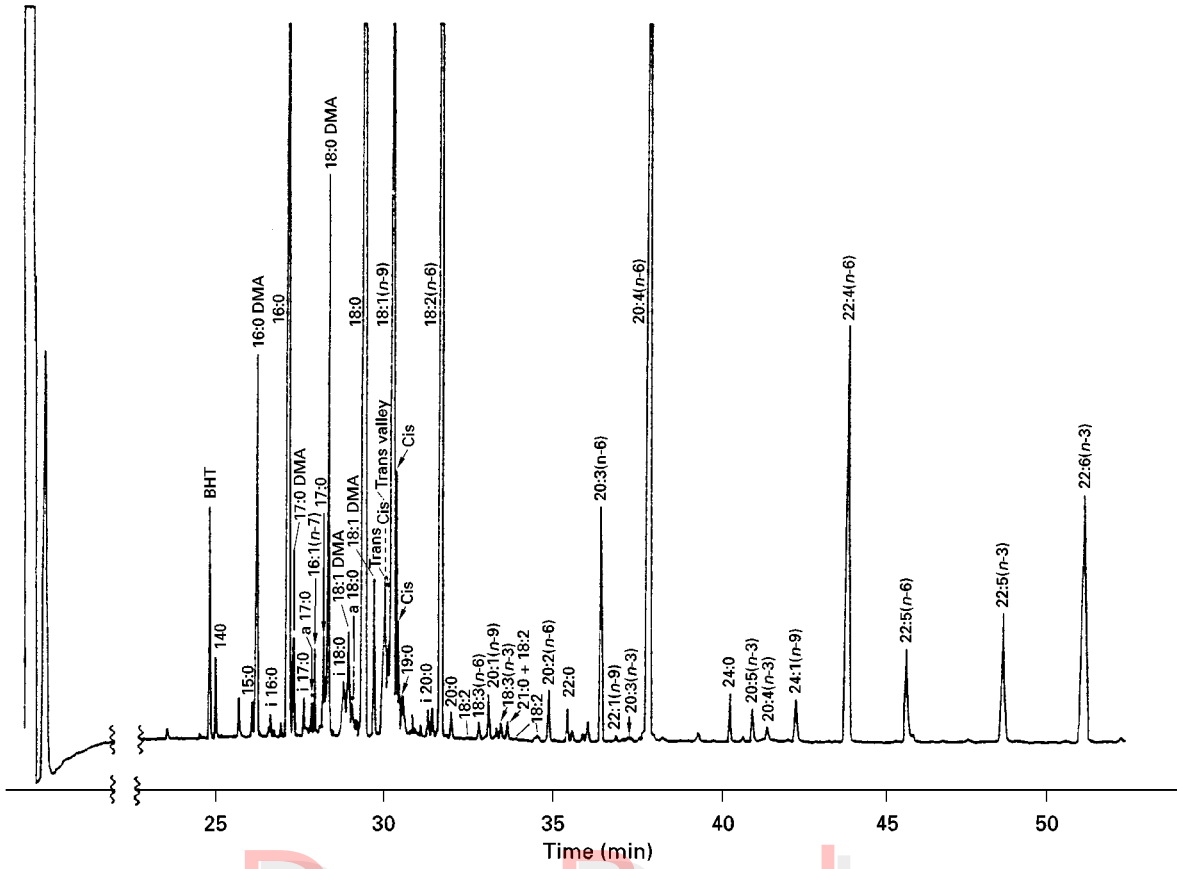

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Figure 9 Polar capillary GC of fatty acid methyl esters and dimethylacetals of human erythrocyte membranes. Peaks are identified by short-hand notation as indicated in figure. GC conditions: column, 100 m × 0.25 mm i.d., 0.2 μm deactivated cyanopropylsiloxane (SP 2560, Supelco); temperature, 80°C (hold 2 min) then 8°C min^{-1} to 220°C and hold for 32 min; carrier gas, helium; split injection 1 : 30. (Reproduced with permission from Alexander LR, Justice JB and Madden J (1985) Fatty acid composition of human erythrocyte membranes by capillary gas chromatography mass spectrometry. *Journal of Chromatography and Biochemical Applications* 342: 1–12.)

isomers were resolved as TMS ethers, PFB esters. The fourth regioisomer, 5,6-DHET, was resolved after being converted to a δ-lactone. The regioisomers of DHET were resolved as the (bis)-TBDMS, PFB esters on a 60 m nonpolar column operated isothermally at 300°C. The 8,9-DHET was followed by 11, 12-DHET, which was followed by 14,15-DHET.

TBDMS ethers have been used for GC-MS separation and identification of synthetic secondary hydroxy fatty acid isomers with carbon chain lengths of 16–20. With the exception of the spontaneous formation of γ- and δ-lactones of C_4-OH and C_5-OH fatty acids, the TBDMS ethers of all hydroxy fatty acid methyl esters in a mixture are readily identified by GC-MS, yielding information on chain length, location of the hydroxyl group and degree of unsaturation. The method has been applied to the identification of a complex mixture of bovine skim milk hydroxy fatty acids, of which 19 were newly identified. The separations were performed on a 30 m column coated with 100% methylsilicone. Hydroperoxides are not sufficiently stable for GC analysis. Therefore, the fatty acid ester hydroperoxides are reduced to the corresponding alcohols with triphenylphosphine and transmethylated with sodium methoxide before GC analysis. A 20 m × 0.32 mm i.d. DB-1 column has been used for GC-MS analysis of linoleate and arachidonate peroxidation products. The hydroxy fatty acid methyl esters were isolated by TLC and converted into TMS ethers. By a combination of normal-phase HPLC and GC-MS, the linoleate oxidation products were identified as 9- and 13-OH derivatives and those arising from arachidonate oxidation as the 5-, 8-, 9-, 12- and 15-hydroxyeicosatetraenoates.

Among the free radical catalysed peroxidation products of arachidonic acid, prostaglandin-like compounds named isoprostanes have been recognized which arise independent of the cyclooxygenase enzyme. The evidence has been reviewed for the natural occurrence of these compounds, their mechanism of formation and methods of determination. They have

GC properties similar to those of prostaglandin $F_{2\alpha}$, with which they may be confused. GC-MS has been recently employed for the measurement of plasma isoprostanes.

Prostaglandins The prostaglandins and thromboxanes are cyclo-oxygenase metabolites of arachidonic acid, which are involved in numerous pathophysiological pathways. High resolution GC in combination with mass spectrometry is generally recognized as the most specific and reliable method for qualitative and quantitative analysis of prostanoids. In fact, the use of high resolution GC is mandatory when the stable cyclo-oxygenase metabolites of arachidonic acid have to be analysed as a group. The application of GC-MS techniques to the analysis of prostaglandins and related substances has been reviewed, as well as the application of GC-MS to the analysis of other oxygenated fatty acids. Electron capture GC-MS analysis of the PFB-TMS derivatives has provided the most sensitive method for molecular mass determination. This method, however, does not provide structural information and even low energy collision-induced dissociation (CID) of the carboxylate anions from these derivatives does not yield structurally significant ions.

Figure 10A shows by means of authentic standards that a complete separation of all major metabolites of arachidonic acid via the cyclo-oxygenase pathway can be obtained in a relatively short time (30 min) using a 30 m capillary column of OV-101–OV-17 (8 : 2). The selected ion monitoring profiles of the prostaglandins and thromboxanes were obtained with the PFB–MO–TMS derivatives at 220°C with helium as carrier gas. Figure 10B shows a chromatogram of the same substances detected by electron capture. A critical aspect of the application of high resolution GC in routine analyses of prostaglandins is that it requires greater purification of biological material.

Structural Identification

The location of double bonds and other modifications of the fatty chains is required for a complete characterization of the structure of the fatty acids. GC-MS techniques approach this problem by chemical modification prior to analysis.

Double-bond isomers Derivatization at the double bond to oxygenated compounds (carbonyl compounds and vicinal diols) leads to distinctive fragmentation patterns that allow determination of the points of unsaturation or epoxidation but this method leads to the formation of highly polar derivatives and an increase in molecular weight, which makes it unsuitable for the analysis of polyunsaturated fatty acids.

An alternative technique is based on derivatization of the terminal carboxylic group (remote site modification), whereby fatty acids are converted to *N*-acyl

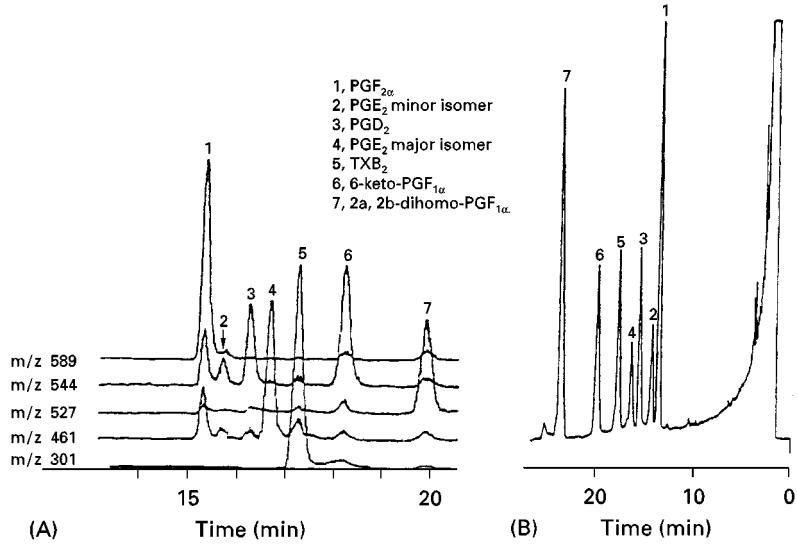

Figure 10 Polar capillary GC and selected ion monitoring (SIM) of authentic prostaglandins and major metabolites of arachidonic acid as PFB-MO-TMS derivatives. (A) SIM; (B) high resolution GC with electron capture detection. Peak identification is given in the figure. GC conditions: column, 30 m × 0.3 mm i.d., dimethylsiloxane/50% phenylmethylsiloxane 8 : 2 (OV-1/OV-17, Ohio Valley); temperature, 220°C isothermal; carrier gas, helium. (Reproduced with permission from Chiabrando C, Noseda A and Fanelli R (1982) Separation of prostaglandins and thromboxane B_2 by high resolution gas chromatography coupled to mass spectrometry or electron-capture detection. *Journal of Chromatography* 250: 100–108.)

pyrrolidides or picolinyl (3-hydroxymethylpyridinyl) esters that easily stabilize the ions containing the double bonds or substituents of the fatty chain. The pyrrolidides have been studied most often for the analysis of natural samples, but recently have been overtaken by the picolinyl esters or 4,4-dimethyloxazoline (DMOX) derivatives. Although distinctive modes of fragmentation can be obtained, the interpretation of spectra becomes substantially more difficult if the number of double bonds is greater than four. The picolinyl esters of isomeric unsaturated fatty acids give more abundant diagnostic ions than the pyrrolidides. **Figure 11** shows the capillary GC separations obtained with the picolinyl and pyrrolidide derivatives of pig testis fatty acids on OV-101 and BP-20 liquid phases, respectively. On the nonpolar methylsilicone (OV-101) column, the more unsaturated picolinyl esters emerge ahead of the less unsaturated and saturated fatty acids of the same acyl carbon number. On the polar BP 20, the pyrrolidide derivatives emerge in order of increasing unsaturation. The picolinyl esters require column temperatures about 50°C higher than methyl esters. The picolinyl esters are resolved according to number of double bonds on polar phases of high temperature stability, such as BPX-70 or Supelcowax 10, but fattty acids of more than 20 acyl carbons may cause difficulty.

It has been shown that the DMOX derivatives of fatty acids are only slightly less volatile than the corresponding methyl esters and that they can be subjected to GC analysis on polar capillary columns to give resolutions based on degree of unsaturation. The GC separation has been carried out with the DMOX derivatives of fatty acids of rat testis and fish oil on a nonpolar methylsilicone (SE-54) column, from which the more unsaturated derivatives emerged ahead of the less unsaturated ones of the same acyl carbon number.

The picolinyl esters and DMOX derivatives can be separated by reversed-phase or argentation HPLC before GC-MS analysis, which is important for the detection of minor components in natural samples. These derivatives have high volatility and their mass spectra show easily recognizable diagnostic peaks for determination of the position of unsaturation. Tables have been prepared of characteristic ions in EI mass spectra of DMOX derivatives of 22 unsaturated fatty acids. The DMOX derivatives have been employed for successful identification of Δ5-unsaturated polymethylene-interrupted fatty acids from conifer seed oils. Most analysts now prefer either picolinyl esters

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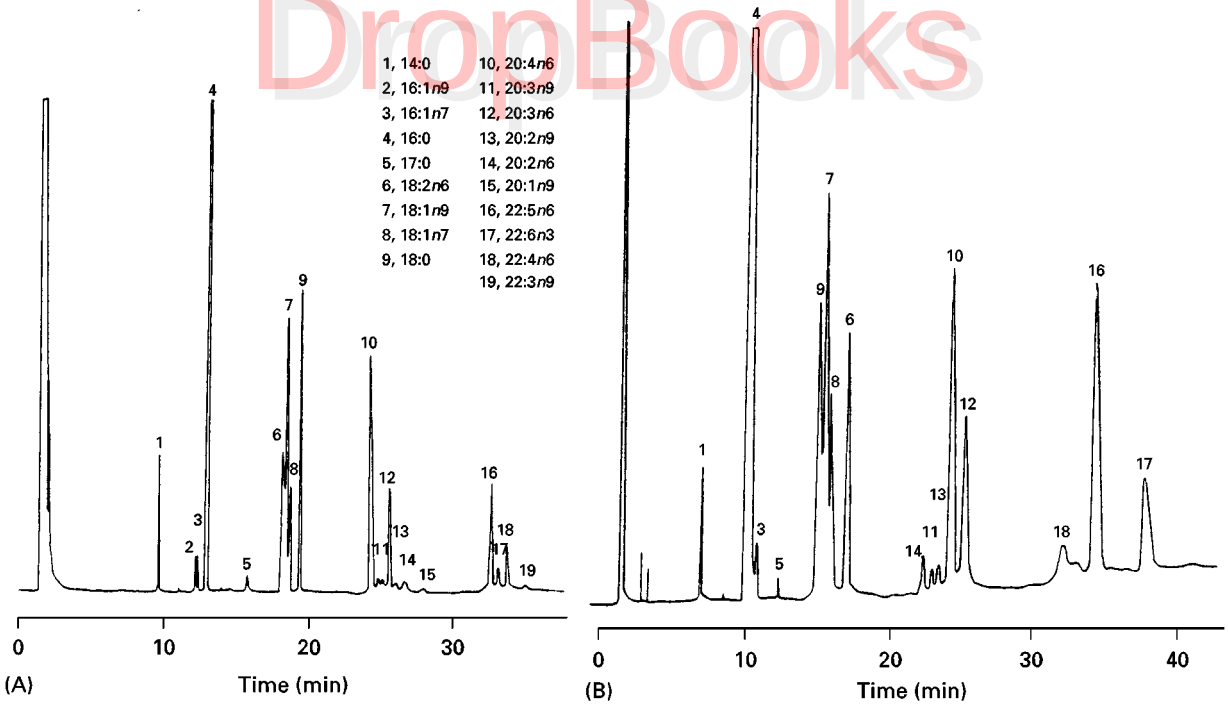

Figure 11 Capillary GC of (A) picolinyl and (B) pyrrolidide derivatives of pig testis fatty acids on nonpolar and polar phases. Peak identification is given in the figure. GC conditions: (A) column, 50 m × 0.22 mm i.d., methylsilicone (OV-101, Ohio Valley); temperature, programmed from 195°C (hold 3 min) then 1°C min^{-1} to 235°C; carrier gas, helium; (B) column, 12 m × 0.22 i.d., polyethylene glycol (BP 20, SGE); temperature, programmed from 190°C (hold 3 min) then 1°C min^{-1} to 230°C; carrier gas, helium. (Reproduced with permission from Christie WW, Brechany EY, Johnson SB and Holman RT (1986) A comparison of pyrrolidide and picolinyl ester derivatives for the identification of fatty acids in natural samples by gas chromatography-mass spectrometry. *Lipids* 21: 657–661.)

or DMOX derivatives for structural studies on fatty acids, but the pyrrolidides also provide informative mass spectra in many instances.

***cis*/*trans*-Isomers and conjugated acids** Identification of *cis*/*trans* isomers of unsaturated fatty acids cannot usually be achieved by GC-MS without reference substances. Satisfactory separations of the geometric isomers from margarine fatty acids and 44 synthetic C_{18} unsaturated fatty acids have been obtained by GC on a 60 m capillary column coated with 100% cyanoethylsilicone (SP-2340). Overlaps occur between different double-bond systems and between some mono- and diethylenic, as well as between di- and triethylenic fatty acids. Because of these overlaps, the isomers cannot be determined by GC alone on the SP-2340 or on any other cyanosilicone phase. Prefractionation by silver ion TLC or HPLC is necessary. The GC separation of the *cis*- and *trans*-acids has been reported in milk samples using a 100 m capillary column coated with bis-cyanopropyl-32% dimethylsiloxane (SP-2560). The authors were able to calculate the total *trans* acid content by adding the relative percentage of all *trans* monounsaturated fatty acids and *cis*/*trans* linoleate and total conjugate dienes in the milk chromatograms. SP-2560 capillary columns were used to conduct an extensive investigation of the effect of various methods of methylation upon the estimation of the conjugated fatty acid content of bovine milk. Acid-catalysed methods caused extensive isomerization of conjugated dienes and formed allylic methoxy artefacts and are therefore not recommended for this purpose. Base-catalysed methods caused no isomerization of conjugated dienes and the formation of artefacts. GC–Fourier transform infrared–MS (GC/FTIR/MS) has been used to identify fatty acid methyl esters and differentiate between *cis*/*trans* isomers. In the FTIR spectra *cis*/*trans* isomers are identified by analysis of bands arising from C-H out-of-plane bending: for both fatty acid methyl esters and DMOX derivatives *cis*-1,2-disubstituted double bonds give a strong band near 720 cm^{-1} and the corresponding *trans* isomers near 967 cm^{-1}. A greatly improved resolution of individual *trans*-18:1 isomers has been reported by capillary GC on a 100 m cyanopropyl polysiloxane (CP-Sil 88) column.

Epoxides The picolinyl esters are also adequate for the location of epoxide functions in linoleic, arachidonic and docosahexaenoic acids. The electron impact (EI) mass spectra of these derivatives show a molecular ion and a sequence of peaks, with two characteristic abundant ions that result from formal cleavage of the carbon–carbon bonds at the oxirane ring. Both these ions retain the ester group. This fragmentation pattern allows the unequivocal identification of the separate epoxide isomers. The picolinyl esters of the epoxides are resolved on a 30 m methylsiloxane (DB-1) column, temperature-programmed from 220 to 300°C. These picolinyl esters provide characteristic GC-MS profiles that allow differentation of the isomeric epoxides at nanogram levels.

Branched-chain and cyclic acids The methyl esters of branched and cyclic fatty acids are usually eluted ahead of their normal-chain counterparts. The methyl branched acids may be confused with straight-chain odd-carbon number isomers. Long (50 m) capillary columns with nonpolar (5% phenylmethylsilicone) stationary phases are preferred and cyclopropane acids are resolved from their monounsaturated counterparts. The position of the methyl branching and cyclopropane ring is not readily determined by GC-MS with EI even after preparation of pyrrolidide and picolinyl derivatives, although exceptions are known. Only small differences are seen among the DMOX derivatives, but they may be sufficient for distinction between close isomers.

It has been shown that low-energy CID of the molecular ions of fatty acid methyl esters obtained by electron ionization (70 eV) in the tandem quadrupole mass spectrometer yield a regular homologous series of carbomethoxy ions. This method can be used to determine methyl (or alkyl) branching positions, as shown by enhanced radical site cleavage at the alkyl branching positions of several methyl esters, including phytanic acid, isomethyl and anteiso-methyl branched acids and tuberculostearic acid. Analyses of various stable isotope variants support the hypothesis of alkyl radical migration to the carboxy carbonyl oxygen atom, with subsequent radical site directed cleavage, either with or without a cyclization event.

GC-MS Analyses of Stable Isotope-Labelled Lipids

In addition to identification of structures, GC-MS also provides the distribution and content of the stable isotope, both natural and enriched. Highly sensitive and reproducible estimates have been obtained for the isotopomer distribution by GC combined with isotope ratio mass spectrometry (isotopomer analysis). Furthermore, the utilization of appropriately labelled isotopic homologues as internal standards for the solute to be measured has greatly improved quantification by GC.

Isotopomer Analyses

Mass isotopomer distribution analysis (MIDA) uses stable isotopes with quantitative mass spectrometry. It is based on the principle that the isotope distribution or labelling pattern of a fatty acid (e.g. palmitic acid) synthesized from an isotopically perturbed monomeric precursor pool conforms to a binominal expansion. The proportion of unlabelled, single-labelled, double-labelled, and so on, molecular species of a fatty acid is a function of the probability that each precursor subunit is isotopically labelled in the fraction of newly synthesized fatty acid present. The advantages and disadvantages of this approach in studies of lipid metabolism have been discussed in the literature. Analogous combinatorial probability methods have long been used to measure endogenous synthesis of fatty acids. The analytical data for the biosynthesis of palmitic acid from [^{2}H-ethanol], [^2H_2O] or [^{13}C-acetate], for example, are provided by GC-MS analysis of the palmitic acid methyl ester and the methyl esters of any other fatty acids of interest. In this method every newly synthesized molecule is counted and the total synthesis calculated by summation of the newly formed molecules.

Definitive Methods

One of the major applications of GC-MS is in quantitative analysis with stable isotope dilution and selected ion monitoring. A known amount of a stable isotope-labelled variant of the molecule to be measured is added to the biological sample as an internal standard, followed by extraction, purification and chemical derivatization. The ions specific for the substance and the internal standard are then monitored. The ratio of the two ion abundances provides the quantitative measurement, which is independent of absolute sensitivity. The superiority of this approach has been demonstrated in the quantitative GC-MS analysis of prostaglandin $F_{2\alpha}$ in a biological extract using a deuterated derivative. Examples of this approach are provided by the highly sensitive measurement of thromoboxane B_2 (TXB_2). Another group based their method on the use of low-blank ($^1H < 0.2\%$) tetradeuterated 18,18,19,19-$^2H_4$ TXB_2, which they synthesized as the internal standard. After purification and HPLC, the samples were derivatized to give an open chain derivative of TXB_2, a PFB–MO–TMS ether derivative, most suitable for negative ion chemical ionization mass spectrometry. In the selected ion monitoring mode, the detection limits per injection for pure standards and biological samples were 10 and 30 pg, respectively. GC was carried out on a 50 m × 0.25 mm i.d. bonded SE-30 column. An antibody-mediated extraction method for GC-MS analysis of thromoboxane A_2 (TXA_2) urinary metabolites has been reported. An antibody (Ab) raised against TXB_2 (35% cross-reacting with 2,3-dinor-TXB_2) was coupled to CNBr-activated Sepharose 4B (Se) and used as a stationary phase for simultaneous extraction of both compounds from urine. Quantification was performed by GC-negative chemical ionization-MS, monitoring the carboxylate ion. The GC was performed on a 26 m cyanopropyl siloxane (CP-Sil 5CB) column.

The vasoconstrictor and platelet activator TXA_2, the predominant product of arachidonic acid in the platelet, is a very reactive substance. It is rapidly converted by nonenzymatic hydrolysis to the stable TXB_2. Detailed GC-MS analyses of the PFB–MO–TMS ether derivatives of the thromboxanes and their major metabolites have been described.

GC-MS with selected ion monitoring has been used to demonstrate the global changes in the prostaglandins and thromboxanes in rat circulation after administration of arachidonic acid. **Figure 12** illustrates the measurements of the prostaglandins with multiple deuterium isotope dilution involving separation of pentafluorobenzyl esters, *O*-methyl oximes and TMS ether derivatives by high resolution GC and specific detection by NICI-MS in the selected ion mode. The selected ion monitoring profiles show the detection of reference prostaglandins (d_0 and d_4) and their 15 K and 15 KD metabolites: top chromatogram, total ion current; other chromatograms, selected ion mass chromatograms.

GC-MS-MS analyses

In GC-MS-MS, a specific ion is selected from the initial mass spectrum. The selected parent ion is allowed to enter a field-free reaction region, where it may undergo unimolecular or CID. The resulting fragments are analysed by a second mass spectrometer, which provides the MS-MS spectrum. Although commonly referred to as MS-MS, tandem mass spectrometric analysis is usually MS-CID-MS. The application of GC-MS-MS for the analysis of long chain carboxylic acids and their esters has proved enormously successful.

Conclusion

The complex nature and large number of molecular species with similar physicochemical properties make GC the preferred method of lipid analysis. GC provides the highest resolution and the shortest analysis time compared to other chromatographic techniques. GC with FID is highly sensitive and readily automated. Its applicability is limited by high temperature

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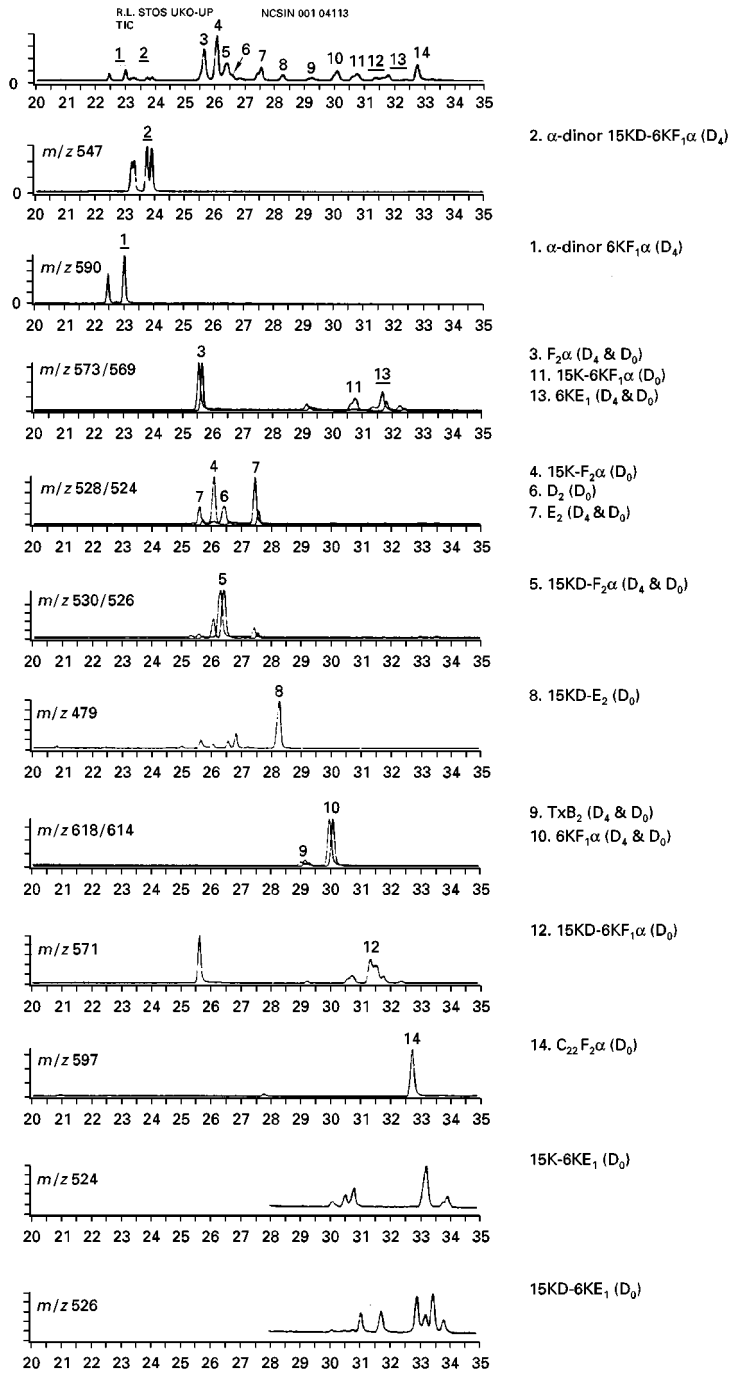

Figure 12 GC-negative chemical ionization-MS with selected ion monitoring of reference prostaglandins (D_0 and D_4) and their 15 K and 15 KD metabolites. Peak identification is given in the figure. GC conditions: column, 60 m × 0.2 i.d., 0.25 μm methyl silicone (DB-1, J & W); temperature, programmed from 100°C (hold for 2 min) to 280°C at 30°C min^{-1}; carrier gas, hydrogen. (Reproduced with permission from Pace-Asciak, 1987.)

stability and low volatility of the analytes, which may be overcome by enzymic and chemical modification of the samples. With appropriate strategy, automated GC provides a precise tool for the resolution and quantification of lipids ranging from fatty acids to triacylglycerols. Although appropriate GC strategy also provides identification of unknowns in relation to standards, this must remain tentative unless combined with mass spectrometry. GC-MS of appropriate derivatives of fatty acids provides details of the molecular structure of the fatty acids and their esters. With continued development of liquid phases of increased thermal stability and lipid derivatives of increased volatility and informative mass spectra, GC

and GC-MS is likely to remain the method of choice for analyses of molecular species of most lipid classes for the immediate future.

See Colour Plate 103.

See also: **II/Chromatography: Gas:** Column Technology; Derivatization. **III/Lipids:** Liquid Chromatography; Thin-Layer (Planar) Chromatography.

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DropBooks

Liquid Chromatography

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Introduction

Natural lipids consist of complex mixtures of molecular species, which are found in association with cell membranes, lipoproteins and other subcellular structures. The composition differs among different cell and tissue types, reflecting the function of lipids in these body structures. Much experimental effort and imagination has been expended in determining the exact composition of lipid species during dietary alterations and physiological activity.

The complex nature of natural lipids and their high solubility in organic solvents makes the separation and isolation of individual lipid classes and molecular species by most physical methods difficult or impossible. Only chromatographic methods have proven suitable for this. The earlier thin-layer and gas chromatographic routines have been complemented in recent years by high performance liquid chromatography (HPLC), which has proven to have nearly universal applicability.

Principles of Liquid Chromatography

Normal-phase Columns and Solvents

Normal-phase HPLC provides resolution based on the overall polarity of the lipid molecules. The